Thioredoxin — a fold for all reasons

The thioredoxin fold is a characteristic protein structural motif that has been found in five distinct classes of proteins that have the common property of interacting with cysteine-containing substrates.

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The thioredoxin fold, named after the protein in which it was first observed, is a distinct structural motif consisting of a four-stranded β-sheet and three flanking α-helices. It has been identified in the three-dimensional structures of proteins from five classes: thioredoxin [1,2] (and see accompanying review article on thioredoxin structure in this issue of *Structure* [3]), glutaredoxin [4–7], glutathione S-transferase [8–11], DsbA [12] (the protein that catalyzes disulphide formation *in vivo* [13,14]) and glutathione peroxidase [15].

The thioredoxin protein is a small ubiquitous molecule classified as a general protein disulphide reductant [16]. The *Escherichia coli* oxidized form was the first thioredoxin structure solved [1,2], and it was found to consist of a single domain with a central five-stranded β-sheet with four flanking α-helices and a dithiol/disulphide group in the active site, protruding from the protein surface. Thus, the thioredoxin fold that is common to the five protein families is somewhat smaller than thioredoxin itself, having one less β-strand and one less α-helix.

The sequence identity between the five protein classes does not suggest a close structural similarity. Moreover, there is no catalytic or biological function common to all five proteins. There is, however, a functional similarity between three of the five; thioredoxin glutaredoxin, and DsbA are all redox proteins (although DsbA is a disulphide oxidant rather than a reductant like the other two), and all three share a Cys-X-X-Cys active-site motif (where X is any amino acid). Interestingly, the residues between the two cysteines at the active sites are quite distinct in the thioredoxin (Cys-Gly-Pro-Cys), glutaredoxin (Cys-Pro-Tyr-Cys) and DsbA (Cys-Pro-His-Cys) protein classes, although they are highly conserved within each family.

Neither of the two remaining thioredoxin fold-containing proteins has the Cys-X-X-Cys motif of the redox proteins. However, both proteins interact with the cysteine-containing substrate, glutathione. Glutathione peroxidase interacts with glutathione via a selenocysteine residue using the substrate as an electron donor to catalyze hydroperoxide reduction. The interaction between glutathione S-transferase and the sulphydryl group of glutathione is mediated via a conserved tyrosine residue, in this case to catalyze the transfer of glutathione to electrophilic groups of cytotoxic compounds.

Although structurally related by their thioredoxin fold, each of these five proteins forms a separate and distinct family. The only other common factor that unites these proteins is cysteine chemistry: all interact with substrates that possess a thiol or a disulphide group.

The thioredoxin fold unravelled

The architecture of the thioredoxin fold has been described previously by Eklund *et al.* [17] in a paper that compares the structures of thioredoxin and glutaredoxin. The β-sheet and α-helices of the fold can be subdivided into an N-terminal βαβ motif and a C-terminal ββα motif, connected by a loop of residues that incorporates a third helix (Fig. 1). Because the thioredoxin fold is a substructure of thioredoxin, the nomenclature for strands and helices of the thioredoxin fold is different to that used for the structure of the thioredoxin protein.

The β-strands from the N-terminal motif, β1 and β2, run parallel, whereas those from the C-terminal motif, β3 and β4, are antiparallel (Figs 1,2). Together, the two regions form a β-sheet of parallel and antiparallel β-strand pairs that is characteristic of the thioredoxin fold proteins. Also characteristic is the arrangement of the α-helices around the central β-sheet. Helices α1 and α3, from the N- and C-terminal motifs, respectively, line up in a parallel fashion on one side of the sheet. The α2 helix that connects the two motifs is located on the opposite side of the β-sheet to the α1 and α3 helices and is oriented perpendicular to them (Figs 1,2).

On alignment of the structures of the thioredoxin fold-containing protein structures, it is apparent that the atom from each protein that interacts with the substrate cysteine/cystine is positioned in the same relative location in space (Fig. 3). This is perhaps not unexpected for the three redox proteins (thioredoxin, glutaredoxin and DsbA) that share a common redox-active disulphide/dithiol group, located at the N terminus of the α1 helix of the thioredoxin fold. However, the equivalent substrate-interacting residues of the other two proteins are not cysteines and are located at other points in the thioredoxin fold: the selenocysteine of gluthathione peroxidase is found in the loop just prior to the α1 helix of its thioredoxin fold and the catalytic tyrosine of glutathione S-transferase is in strand β1 (Fig. 4). Given that these active-site residues are different in the five classes and arise from different regions

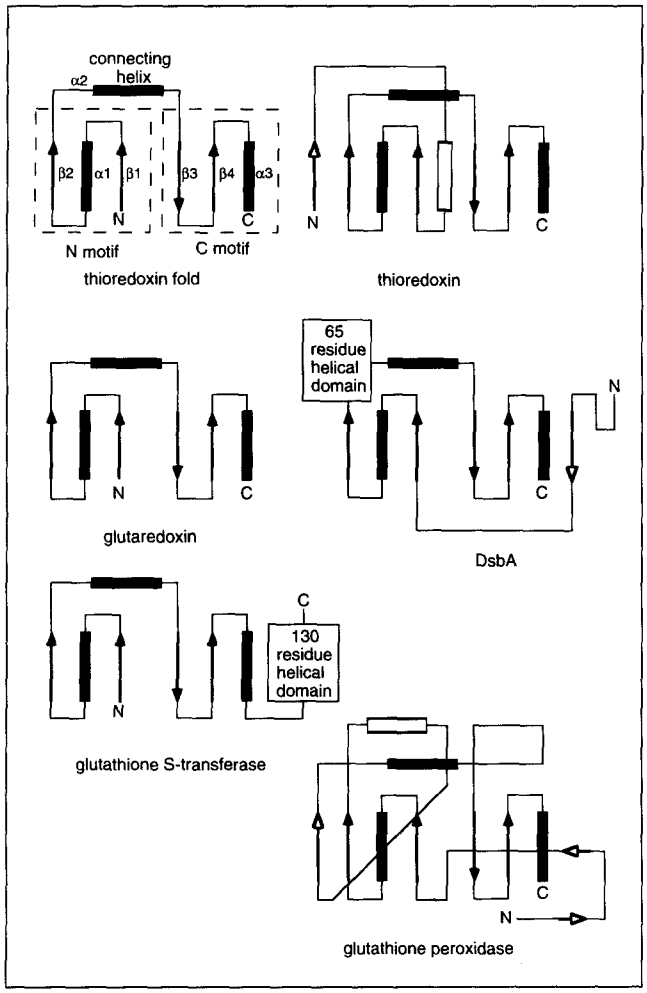

Fig. 1. Architecture of the thioredoxin fold proteins. β-sheet strands are drawn as arrows and α-helices as rectangles. The thioredoxin fold archetype is shown top left, with dashed lines indicating separation of the fold into N-terminal (βαβ) and C-terminal (ββα) motifs connected by the α2 helix. Also shown are equivalent features in thioredoxin, glutaredoxin, DsbA, glutathione S-transferase and glutathione peroxidase. Helices and strands that are not part of the thioredoxin fold are drawn in outline only, or identified as separate domains.

of the thioredoxin fold, it is remarkable that the substrate-interacting atoms correspond so well.

The thioredoxin fold *in situ*

The thioredoxin fold comprises about 80 residues, but each of the proteins containing it has extra residues in addition to the fold (Fig. 1). Glutaredoxin (87 residues) and thioredoxin (108 residues) are single-domain monomeric proteins: glutaredoxin has little structure in addition to the basic thioredoxin fold, whereas thioredoxin has an extra β-strand and α-helix at the N terminus. DsbA (189 residues) is also a monomer, but it has two distinct domains — a thioredoxin fold and an α-helical domain, which is inserted into the thioredoxin fold at a point between β2 and α2. Glutathione S-transferase is a homodimer with each subunit comprising 217 residues in two domains; in this case the helical domain is connected to the C terminus of the thioredoxin fold. Finally, the 198-residue single-domain subunit of glutathione peroxidase forms a homotetramer. Most of its additional residues are inserted at the same point as those in DsbA, that is, between β2 and α2. Other additional residues in glutathione peroxidase are inserted between α2 and β3. Instead of forming a separate domain as in DsbA, the inserted residues of glutathione peroxidase wind around the thioredoxin fold to produce the tetramer interface, part of which forms a fifth strand in the thioredoxin fold β-sheet (Fig. 1). Thioredoxin, DsbA and glutathione peroxidase all have additional residues at the N terminus of the thioredoxin fold; in thioredoxin and DsbA these form a fifth strand in the β-sheet, although in DsbA this is at the opposite end of the sheet to that of thioredoxin and glutathione peroxidase (Fig. 1).

Variations on a theme

The presence of a fifth strand and additional residues at the N terminus of the thioredoxin fold in thioredoxin, glutathione peroxidase and DsbA, in addition to other structural differences (Table 1), serve as a basis for structurally separating these three proteins (which I have termed the TRX proteins) from glutaredoxin and glutathione S-transferase (the GRX proteins). In general, the strands and helices in the thioredoxin fold of the GRX proteins are shorter than those of the TRX proteins.

The GRX proteins also have a significant twist in strand β1 that is not present in the TRX proteins (Fig. 3). This is due to a main-chain ϕ angle of close to 180° for residue 6 in the GRX proteins (glutaredoxin Gly6 ϕ=+173°,

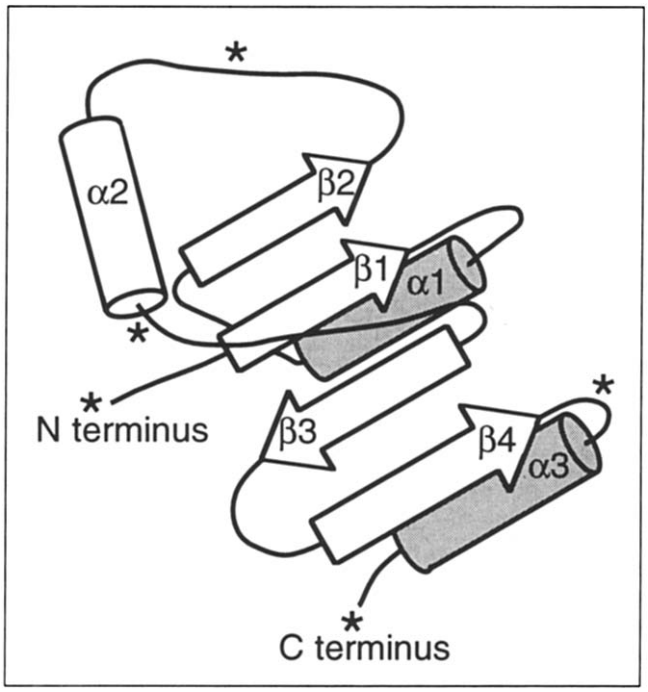

Fig. 2. The thioredoxin fold. The placement of the helices with respect to the central β-sheet is shown. The shaded helices, α1 and α3, are behind the sheet in this orientation, whereas helix α2 is in front of the β-sheet. Points in the structure at which insertions of residues are found are indicated by asterisks.

glutathione S-transferase Tyr6 ϕ=−177°). The equivalent residues in the TRX proteins have ϕ angle values of −145° for Trp28 in thioredoxin, −153° for Phe26 in DsbA and −120° for Val41 in glutathione peroxidase. Tyr6 of glutathione S-transferase is the catalytically important tyrosine residue that interacts with the thiol group of the glutathione substrate. The main-chain ϕ angle for this residue may be necessary to orient the tyrosine side chain into the correct position for interaction, as the side chains of the equivalent residues in the TRX proteins point in the opposite direction — into the interior of the protein. However, this difference may also be due to sequence variation, because the equivalent residues of the TRX proteins are more hydrophobic (Trp, Val, Phe, respectively, for thioredoxin, glutathione peroxidase and DsbA).

Finally, for the two GRX proteins, there is a direct connection (no loop) between strand β4 and helix α3,

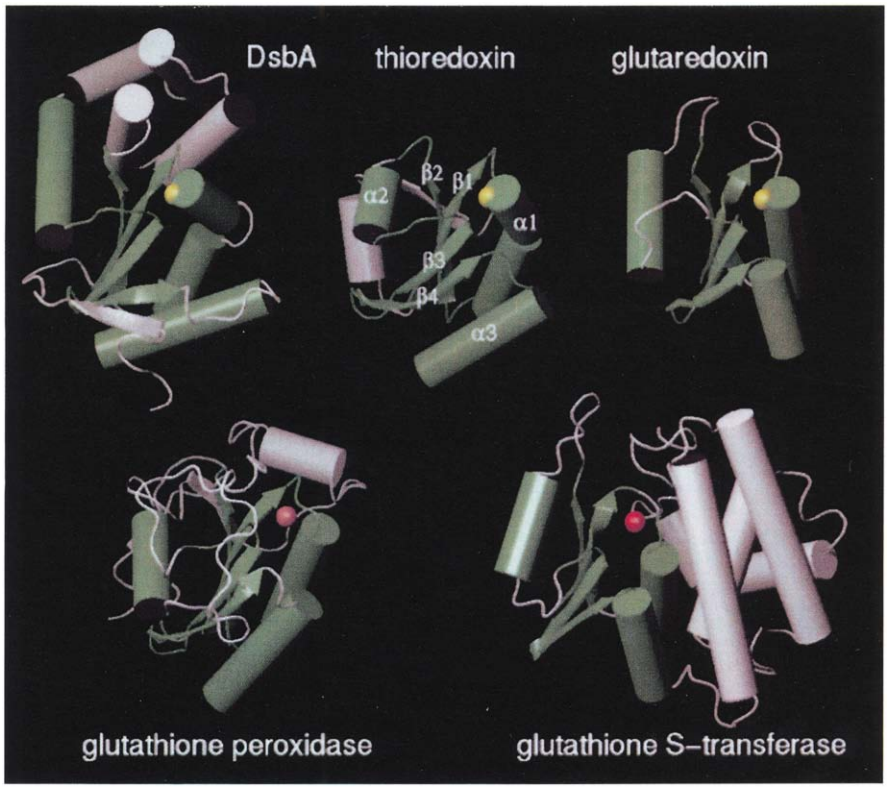

Fig. 3. Structural comparison of the five thioredoxin fold proteins. Structures of DsbA, thioredoxin, glutaredoxin, glutathione peroxidase and glutathione S-transferase are depicted, with those parts of each structure that form part of the thioredoxin fold motif shown in green (shown in same orientation as fold in Fig. 2). Other structural features are shown in grey. The atoms that interact with the cysteine residue of the substrate are depicted as coloured spheres — yellow indicating the sulphur atom of the more N-terminal cysteine of the Cys-X-X-Cys motif in thioredoxin, glutaredoxin and DsbA, pink indicating the selenocysteine selenium of glutathione peroxidase and red indicating the tyrosine hydroxyl oxygen of glutathione S-transferase. The figure was generated with the program O [18] using the highest resolution structures available of the five wild-type proteins, taken from the Brookhaven protein data bank; where more than one structure of the same or similar proteins was available, these were used to check the validity of conclusions. 2trx for oxidized *E. coli* thioredoxin at 1.68 Å, R=16.5% [3];1aaz for oxidized bacteriophage T4 glutaredoxin at 2.0 Å, R=21.0% [4];1dsb for oxidized *E. coli* DsbA at 2.0 Å, R=16.9% [11]; 1gp1 for bovine erythrocyte glutathione peroxidase at 2.0 Å, R=17.1% [7] and 1gst for rat liver μ class glutathione S-transferase complexed with glutathione, at 2.2 Å, R=17.7% [9]. The stereochemical quality of the structures was checked with PROCHECK [22].

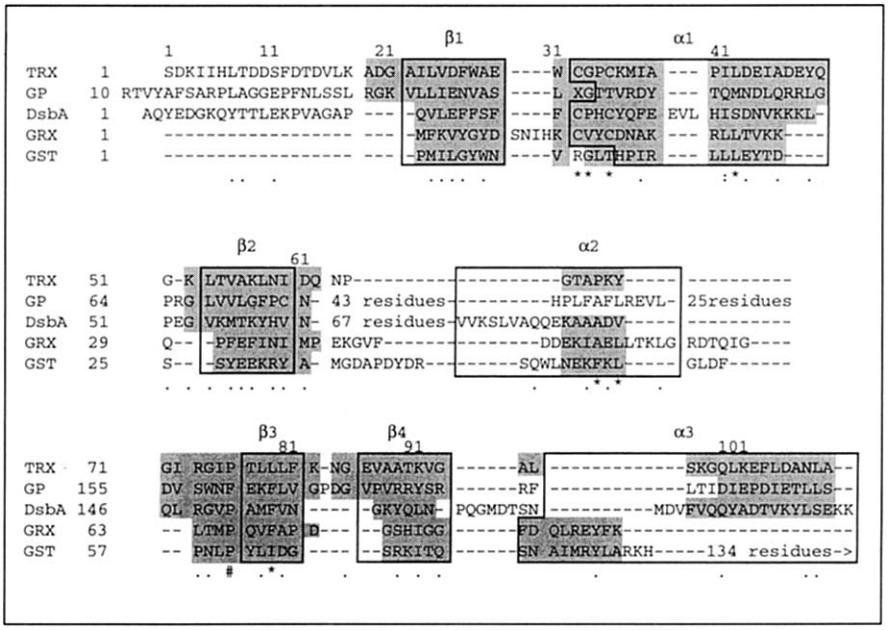

Fig. 4. Sequence comparison of the thioredoxin fold proteins. Sequence alignment of thioredoxin (TRX), glutathione peroxidase (GP), DsbA, glutaredoxin (GRX) and glutathione S-transferase (GST) based on the structural alignment of the thioredoxin folds. Secondary structural features of the thioredoxin fold are boxed in solid lines and labelled β1, α1, etc. Every tenth residue of the thioredoxin sequence is numbered and shading indicates residues that are structurally aligned with thioredoxin, in other words those used to calculate rms differences for the top row in Table 2. A dot (.) below the sequence alignment indicates residues identical in two of the proteins, a colon (:) indicates two pairs of identical residues, an asterisk (*) indicates residues identical in three of the proteins, and a hash (#) indicates residues identical in four of the protein sequences.

Table 1. Structural features of the two subtypes of the thioredoxin fold.

Structural feature	TRX proteins: thioredoxin, DsbA and glutathione peroxidase (no. of residues)	GRX proteins: glutaredoxin and glutathione S-transferase (no. of residues)
Additions at N terminus	20–34	0
Strand β1	8–9	8[a]
Connection between β1 and α1	1–3	5
Helix α1	17–19[b]	11–15
Connection between α1 and β2	2–3	1
Strand β2	8	7
Connection between β2 and α2	4–68[c]	7–10
Helix α2	6–16[d]	10–13[d]
Connection between α2 and β3	2–27[e]	4–6
cis-Pro loop	4[f]	4
Strand β3	5	5
Connection between β3 and β4	0–4	0–1
Strand β4	6–8	6
Connection between β4 and α3	2–8	0[g]
Helix α3	14–19	9–12
Additions at C terminus	0	0–134[h]

[a]This strand incorporates the active-site tyrosine of glutathione S-transferase, and is twisted in both that protein and glutaredoxin but not in the other three proteins. [b]This helix is kinked in DsbA and thioredoxin; it incorporates the CXXC active-site motif of the three redox proteins, and the active-site selenocysteine of glutathione peroxidase. [c]DsbA and glutathione peroxidase have large insertions of residues at this point. [d]This helix is quite variable in all five structures, and can be present as a 3_{10} or an α-helix. [e]Glutathione peroxidase has a large insertion of residues here. [f]Glutathione peroxidase is the only one of the five proteins that does not have a *cis*-Pro at this point. [g]This connection significantly affects the positioning of the α3 helix. [h]Glutathione S-transferase has an additional domain here.

whereas the three TRX proteins have a loop (varying in length from two to eight residues) connecting these two elements of secondary structure (Table 1). This difference in connectivity results in a dramatic difference in the orientation of the α3 helix (Fig. 3). This orientation difference may result in altered substrate specificity in the GRX proteins α3, an idea supported by the fact that the γ-Glu carboxylate of bound glutathione is stabilized in both proteins by the positive charge of the helix dipole of helix α3 [7,11].

Structural comparison

How similar are the thioredoxin folds of the five proteins? This question was answered by carrying out structural comparisons using the least-squares fitting procedures in O [18], by comparing the root mean square (rms) deviation in Cα positions of residues from the thioredoxin folds of the five proteins (Table 2) and by looking at the sequence alignment, based on the structural alignment of the thioredoxin folds (Fig. 4). The results further emphasize the differences between the TRX and the GRX proteins.

Thus, whereas 67–82 residues may be aligned between thioredoxin, glutathione peroxidase and DsbA, this number drops to 44–57 residues when these proteins are compared with the GRX proteins. This is primarily because the α3 helices of the GRX proteins are oriented differently to the α3 helices of the TRX proteins. Furthermore, as the secondary-structure elements of the GRX proteins are generally shorter than those of the TRX proteins, there are fewer residues for alignment between the two subtypes. Comparison of the thioredoxin folds of the two GRX proteins, however, reveals that 61 residues can be aligned with an overall rms deviation in Cα positions of 1.7 Å. This is notable, given that the sequence of glutaredoxin comprises only 87 residues.

Sequence comparison indicates active site similarity

Sequence alignment shows that there are no areas that are conserved in all five proteins. However, two regions of local sequence similarity are found in the three oxidoreductase proteins, thioredoxin, glutaredoxin and DsbA. These two stretches of sequence — Cys32-Gly33-Pro34-Cys35 and Arg73-Gly74-Ile75-Pro76 in thioredoxin — have been shown [7] or are predicted [12,17] to form part of the active site in all three redox proteins.

The first of these sequences incorporates the redox-active disulphide at the N-terminal part of the α1 helix of the redox proteins. In glutathione peroxidase and glutathione-S-transferase, this active-site disulphide is missing and the α1 helix is truncated (Fig. 4). Although the disulphide is absent in these two proteins, this region of the sequence also forms part of the active site. Thus, in glutathione peroxidase, the catalytic selenocysteine that interacts with glutathione substrate can be aligned with the accessible cysteine (Cys32 in thioredoxin) of the redox proteins. Furthermore, whereas glutathione S-transferase does not incorporate an equivalent residue to the cysteine/selenocysteine of the other four proteins, the crystal structure complex between glutathione S-transferase and glutathione [9] shows that the substrate thiol is bound at this same accessible point by an interaction with the conserved Tyr6 hydroxyl of glutathione S-transferase. Clearly, the sequence similarity between the three redox proteins and the structural similarity of all five proteins at this position, suggest that this region of the thioredoxin domain has been conserved or has converged in evolution to interact with a cysteine residue in thiol/disulphide-containing substrates.

The second region of sequence similarity in the redox proteins, termed the *cis*-Pro loop, falls between α2 and

Table 2. Rms differences for Cα atoms of aligned residues in the thioredoxin fold proteins.[a]

	Thioredoxin	Glutathione peroxidase	DsbA	Glutaredoxin	Glutathione S-transferase
Thioredoxin		2.1 Å (82)	1.9 Å (74)	1.8 Å (57)	1.9 Å (54)
Glutathione peroxidase	2.1 Å (82)	–	2.2 Å (67)	2.5 Å (54)	2.8 Å (54)
DsbA	1.9 Å (74)	2.2 Å (67)	–	1.9 Å (44)	2.6 Å (45)
Glutaredoxin	1.8 Å (57)	2.5 Å (54)	1.9 Å (44)	–	1.7 Å (61)
Glutathione S-transferase	1.9 Å (54)	2.8 Å (54)	2.6 Å (45)	1.7 Å (61)	

[a]Number of aligned residues, for each pair of proteins, is given in parentheses.

β3 of the thioredoxin fold. In the structures of these three proteins, this loop of residues is exposed to solvent, and the proline, which is highly conserved in all three protein families, is in the less common *cis*-conformation. A similar *cis*-proline is found in the same region of sequence in glutathione S-transferase (Pro57-Asn58-Leu59-Pro60), but not in glutathione peroxidase (Ser157-Trp158-Asn159-Phe160). Indeed, this region of glutathione peroxidase is structurally different to that of the other proteins and instead of being solvent-exposed, residues Asn159 and Phe160 are buried by strand β4.

The structures of complexes between the substrate glutathione and either glutaredoxin [7] or glutathione S-transferase [8,9] show that many significant interactions are formed with the *cis*-Pro loop. The packing between glutathione and the *cis*-Pro loop is described as a short antiparallel β-sheet [8] and the *cis*-form of the proline is thought to be essential for the formation of the main-chain hydrogen bonds between substrate and protein. The presence of a conserved *cis*-proline in thioredoxin and DsbA suggests that similar main-chain interactions might also be formed with their respective substrates. The conserved sequence and structure of the *cis*-Pro loop, observed in four of the proteins, suggests that this region like the redox active site of the thioredoxin fold, has been preserved or has converged in evolution to form part of a substrate-binding site.

Allowed insertion points of the thioredoxin fold

Certain points in the thioredoxin fold can tolerate insertions without disruption of the overall structure. (These points are indicated by asterisks in Fig. 2.) One such insertion point is at the N terminus, where the TRX proteins all have an extra 20–30 residues relative to the GRX proteins. A second position that permits insertions is after the N-terminal βαβ motif, (that is, after β2). In glutathione peroxidase and DsbA there are huge insertions of 40–70 residues at this position compared with an insertion of only two residues in thioredoxin. Fig. 3 shows that these insertions form a helical domain in DsbA, whereas in glutathione peroxidase they create a meandering loop that forms part of the interface of the tetramer subunits. The GRX proteins also have insertions after β2, but these are of a more moderate size (7–10 residues). Both GRX proteins have small insertions (4–6 residues) after α2, whereas the TRX protein, glutathione peroxidase, has a 25-residue insertion at the equivalent point which also contributes to tetramer interface interactions in this protein.

There are at least two other points in the structure of the thioredoxin fold where insertions are allowed: the connection between β4 and α3 (DsbA has an insertion of eight residues at this point, the other four proteins have either no or two connecting residues), and the C terminus, at which point glutathione S-transferase has a whole domain appended to the thioredoxin fold. In both positions, the insertions may be involved in substrate interactions. In DsbA, the additional residues between β4 and α3 help to form a deep, hydrophobic groove near the active site that may form part of a substrate-binding site [12], and in glutathione S-transferase the extra domain contributes residues that help to stabilize glutathione and the electrophilic substrate [10].

A common ancestor?

The overall structural similarity between the thioredoxin folds in all five proteins and the similarity in the positioning of their important active-site residues is striking, given their functional differences and low sequence identity. Sinning *et al.* [11] proposed a 'glutathione-binding protein' ancestor for glutaredoxin, glutathione S-transferase, glutathione peroxidase and thioredoxin, on the basis of the excellent fit between glutathione S-transferase and glutaredoxin structures, and the slightly lower structural fit to thioredoxin and glutathione peroxidase. However, it is possible that an ancestor of all five proteins may have been less specific in its substrate interactions, interacting with a variety of cysteine-containing ligands, much like thioredoxin.

It is probable that an ancestor protein would have incorporated a Cys-X-X-Cys active site and a *cis*-Pro loop, as sequences similar to these structural units are found in the three redox protein families of thioredoxin, glutaredoxin and DsbA and have been shown to form substrate interactions in the glutaredoxin complex with glutathione [7]. In the case of glutathione peroxidase and glutathione S-transferase the structural units are conserved, even though the sequences in these regions are quite different, and may form part of a substrate-binding site. If these two proteins did have a common thioredoxin-like ancestor, their active-site residues have evolved separately from those in the redox proteins, perhaps to accommodate different functions.

Conclusion

The thioredoxin fold is a motif common to five classes of proteins, that vary in their function and have little structural similarity apart from the fold and a common binding site that interacts with cysteine-containing substrates.

The identifying features of each protein family comprise the points at which inserts and modifications bestow additional properties on the individual thioredoxin fold proteins. These points are the C-terminal domain of glutathione S-transferase; the inserted residues in glutathione peroxidase that allow tetramer formation and the binding of lipid peroxides; and the extra residues and inserted domain of DsbA. Two sub-types of the thioredoxin fold — TRX and GRX — have been identified based on several structural differences.

The conserved positioning and size of insertions, and the preservation of secondary structure and specific regions of sequence can be viewed as criteria to define thioredoxin fold proteins. These criteria can be used to check the reliability of sequence alignment and therefore also the modelling of predicted thioredoxin-like proteins. Such

proteins include protein disulphide isomerase — the eukaryotic and much larger equivalent of DsbA, that has two domains with clear sequence similarity to thioredoxin [19] — and the more recently identified periplasmic thioredoxin [20] and membrane bound thioredoxin [21].

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